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High Epstein-Barr Virus Serum Load and Elevated Titers of Anti-ZEBRA Antibodies in Patients With EBV-Harboring Tumor Cells of Hodgkin's Disease

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Hodgkin's disease is commonly associated with EBV latent infection. The incidence of EBV reactivation (active infection or EBV infection with replicative cycle) was evaluated in a series of 30 patients with untreated Hodgkin's disease (except for one case with chronic lymphocytic leukemia) by quantitation of EBV DNA and titration of anti-ZEBRA antibodies in serum samples. DNA was detected in serum (>2.5 × 10^2 genomes/ml) in 15 of 30 patients and was more frequent in Hodgkin's disease with EBV-positive Reed-Sternberg cells (10/12) than in EBV-negative cases (5/18), ($P < 0.01$). Of interest was the demonstration that viremia correlated well with increased titers of anti-ZEBRA IgG and/or standard serological profiles of EBV reactivation (12/15), ($P < 0.05$). However the lack of EBV replicative cycle in Reed-Sternberg cells (negative for ZEBRA antigen and early antigen BHLF1) suggests that the viral replication occurs in a nonneoplastic cell compartment rather than in tumor cells. The measurement of EBV DNA loads and the titration of anti-ZEBRA antibodies shed new lights on the link between activation of EBV replication and Hodgkin's disease: these serological markers together with the determination of the EBV status of the tumor suggest that replication of the viral genome occurs with a decreased efficiency of the immune system, thus allowing progression of the tumor. ***J. Med. Virol. 57:383–389, 1999.***

KEY WORDS: EBV replication; PCR; ZEBRA (BZLF1); Hodgkin's lymphoma

INTRODUCTION

The growth transforming potential of Epstein-Barr virus (EBV), established for Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC), is now suspected to play a role in other neoplasms such as Hodgkin's disease [Pallesen et al., 1991; Herbst, 1996]. Tissues from 40% of patients with Hodgkin's disease are found to contain EBV genomes, usually within the Reed-Sternberg (RS) cells [Weiss et al., 1989]. In Hodgkin's disease, RS cells are latently infected by EBV, and EBV DNA-positive malignant cells express EBER-1/2 and LMP-1 RNA but are negative for EBNA-2 antigens. Some previous reports have clearly demonstrated that the EBV replicative cycle can occur, although rarely, in Hodgkin's disease [Brousset et al., 1993a, 1993b]. Such a replication involves the expression of the ZEBRA protein (also named BZLF1, EB1, or Zta), which plays a crucial role in the switch from EBV latency to EBV replicative cycle [Miller, 1990]. Thus, the presence in serum of antibodies directed against ZEBRA is considered as a good marker for viral reactivation and was detected in 75%–87% of NPC patients [Joab et al., 1991; Mathew et al., 1994]. EBV replication is usually associated with the release of virions and there is a growing interest for the quantitation of EBV load in the serum [Gan et al., 1994; Yamamoto et al., 1995]. One approach is to use quantitative PCR (QPCR) to determine the actual number of viral ge-

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nomes (viral load) present in the peripheral blood. We have developed an EBV genome quantitation technique based on a methodology previously used in our laboratory [Niveleau et al., 1994] and determined the viral burden in 30 sera of patients with Hodgkin's disease. In the present work, both the titration of anti-ZEBRA antibodies (IgG and IgM) and the level of EBV DNA in serum were evaluated. A good correlation was found between these two biological markers of EBV replication, suggesting that reactivation of EBV or active infection may occur in Hodgkin's disease.

MATERIALS AND METHODS

Case Selection

Thirty cases of untreated Hodgkin's disease were selected and classified according to the Rye classification by combined morphological evaluation and immunochemistry on tissue sections [Chital et al., 1988]. All these cases were negative for human immunodeficiency virus (HIV). Tissue samples were processed routinely, i.e., formalin-fixed and paraffin-embedded. In four cases, frozen material was available.

Immunochemistry and In Situ Hybridization

The presence of EBV was assessed on paraffin-embedded tissue sections by immunochemistry with anti-LMP1 antibodies and by in situ hybridization using EBER1 and EBER2 oligoprobes. Activation of EBV replication was searched by immunochemistry with anti-ZEBRA monoclonal antibodies (clones AZ 125 and AZ 130) on frozen sections using the APAAP method as previously described [Brousset et al., 1993a, 1993b).

Production of Recombinant GST-ZEBRA Protein

The fusion recombinant protein was purified from the supernatant of an *E. coli* culture transformed by the PGEX EB1/ZEBRA plasmid segment after IPTG induction. Briefly, one liter of the culture in Luria-Bertani medium (3 hr at 37°C on rotative shaker) was induced by addition of IPTG (final concentration of 0.5 mM). The bacterial pellet was harvested and lysed for 30 min at 37°C in 5 ml of MTPBS buffer (PBS pH 7.3, 100-mM EDTA, 1% Triton-X100 [V/V], 1 mM phenylmethylsulphonyl fluoride) and 500 μl of 1 mg/ml lysozyme. After sonication, the lysate was submitted to affinity chromatography on glutathione cross-linked beaded agarose. Finally, proteins were eluted by 20-mM glutathione (all chemicals were from Sigma, St Quentin Fallavier, France).

Standard Serological Protocol

Serological profiles by titers are defined as follows. A: reactivation profile = anti-VCA IgG > 640; anti-EA > 40, and anti-EBNA > 160. B: past infection reactivation profile = anti-VCA IgM < 10, 20 < IgG < 160; 5 < anti-EA < 40; 10 < anti-EBNA < 80. N: negative profile = anti-VCA IgM < 10; IgG < 5, anti-EA < 5, anti-EBNA < 5.

Titration of anti-ZEBRA antibodies (IgG and IgM) was performed with an ELISA procedure using recombinant ZEBRA protein as antigen [Brousset et al. 1994]. Patients with anti-ZEBRA antibodies were separated into three groups according to the signal observed (A_{450}): a high-titer group with absorbance > 1 A_{450} unit, an intermediate-titer group giving signals between 0.5 and 1.0 A_{450} unit, and a low-titer group giving signals between 0.5 A_{450} unit and the cutoff value (0.3 A_{450} unit); 398 healthy blood donors were used as controls. Anti-ZEBRA antibodies were detected in 14.8% (59/398), including 6 patients with high antibody titers.

Human Sera and Determination of EBV Load in Serum

Serum samples were stored at –20°C before testing. For PCR detection of EBV, 0.5 ml of serum was concentrated by centrifugation at 10,000 g. Determination of EBV load in serum by PCR ELISA was performed as already described [Niveleau et al., 1994]: EBV DNA was first extracted from serum by a rapid alkaline lysis. Ten μl of 1-M sodium hydroxide was added to 10 μl of serum and incubated at 37°C for 60 min. The mixture was neutralized with 30-μl 0.2-M Tris pH 7.5. The PCR was carried out by using two primers spanning a segment of 186 base pairs, chosen in the *Bam*H1W region of the EBV genome. The primers had the following sequences: 5′-TTTGTCCCCACGCGCGCATA-3′ and 5′-AGGTGGCGTAGCAACGCGAA-3′. They were biotinylated at the 5′ end. Aliquots (10 μl) of serum lysates were subjected to DNA amplification in 100-μl conventional PCR buffer containing 200 μM each of dATP, dTTP, and dGTP and 40-μM 5-methyl-dCTP (Boehringer-Mannheim, Meylan, France) and 40-μM dCTP. The biotinylated primers allowed the denatured amplification products (50 μl/well) to be retained in a microtitration plate in which streptavidin had been covalently linked. After 15 min at room temperature, wells were washed three times with phosphate-buffered saline supplemented with Tween 20 (PBST). Monoclonal antibodies directed against 5-methylcytidine were distributed (50-μl undiluted hybridoma supernatant/well) and incubation was maintained during 30 min at room temperature. Wells were then washed three times with PBST containing 1% bovine serum albumin (PBST-BSA). After incubation with peroxidase-conjugated antimouse Igs (H + L) and washings with PBST-BSA, the substrate was added (3-3′-5-5′ tetramethylbenzidine mixed with hydrogen peroxide, TMB peroxidase EIA substrate kit, BioRad, Richmond, CA) and the A_{655} signal was measured in a microplate reader (model 3550 BioRad). The collected values were compared with calibration curves obtained by serial dilutions of EBV DNA extracted from Namalwa cells (a Burkitt lymphoma cell line containing two copies/cell of type A EBV). Results were expressed as EBV genome equivalents/ml of serum. A cutoff value of 2.5×10^2 EBV genomes/ml was selected, since 4/24 serum samples from healthy blood donors exhibited such a level. Chi-square test was used

TABLE I. Summarized Results Concerning Anti-ZEBRA Antibodies and EBV Load in Serum From 30 Individuals With Hodgkin's Disease, According to the Clinical, Pathological, and Serological Findings[a]

Patient (sex/age)	HD subtype and stage (tumor EBV status)	Anti-ZEBRA IgG titers (absorbance)	Genome equivalents EBV/ml serum	Anti-VCA IgG	Anti-EA IgG	Anti-EBNA IgG
C.L. (M/69)	Chronic lymphocytic leukemia and HD3 IBb (EBV-positive)	2.730 (+)	1.75×10^5	5,120	1,280	<5
E.M. (M/80)	HD3 IVBb (EBV-positive)	0.974 (+)	5×10^5	20	<10	<5
B.M. (M/34)	HD3 IVBb (EBV-positive)	2.378 (+)	2.5×10^7	320	40	5
C.J. (M/67)	HD3 IVBb (EBV-positive)	0.537 (+)	2.5×10^7	640	40	5
R.G. (F/75)	HD3 IBb (EBV-positive)	0.477 (+)	7.5×10^2	80	40	<5
B.R. (M/84)	HD3 IIIBb (EBV-positive)	0.358 (+)	2.50×10^2	160	40	5
P.A. (M/59)	HD3 IAa (EBV-negative)	0.308 (+/–)	5×10^2	160	10	20
R.F. (M/34)	HD3 IIAa (EBV-negative)	0.114 (–)	7.5×10^2	160	40	5
L.M. (F/64)	HD3 IVBb (EBV-positive)	0.165 (–)	5×10^3	640	40	20
B.A. (M/94)	HD3 IBb (EBV-positive)	0.077 (–)	$<2.5 \times 10^2$	640	40	5
T.S. (F/17)	HD3 IVBb (EBV-positive)	0.160 (–)	$<2.5 \times 10^2$	160	<10	80
D.P. (F/71)	HD3 IIIBb (EBV-negative)	0.098 (–)	2.5×10^2	640	20	20
K.A. (F/26)	HD3 IIAa (EBV-negative)	0.050 (–)	$<2.5 \times 10^2$	40	<10	80
M.R. (M/61)	HD3 IIAb (EBV-negative)	0.230 (–)	$<2.5 \times 10^2$	80	<10	20
A.R. (M/80)	HD2 IIAa (EBV-negative)	1.612 (+)	5×10^2	640	40	20
C.F. (F/28)	HD2 IIIAa (EBV-negative)	1.635 (+)	$<2.5 \times 10^2$	320	<10	80
M.J. (M/48)	HD2 IAa (EBV-negative)	1.519 (+)	$<2.5 \times 10^2$	160	<10	80
F.R. (F/78)	HD2 IIBb (EBV-positive)	0.857 (+)	2.5×10^3	40	<10	<5
F.J. (F/71)	HD2 IIIBb (EBV-positive)	0.202 (–)	7.5×10^2	640	20	5
C.L. (M/20)	HD2 IIBb (EBV-negative)	0.080 (–)	5×10^3	160	<10	80
S.B. (F/42)	HD2 IIBb (EBV-negative)	0.161 (–)	5×10^2	40	<10	20
D.S. (F/15)	HD2 IAa (EBV-negative)	0.223 (–)	$<2.5 \times 10^2$	10	<10	<5
D.M. (M/28)	HD2 IIIAb (EBV-negative)	0.144 (–)	$<2.5 \times 10^2$	2,560	40	20
V.M.L. (F/42)	HD2 IIIBb (EBV-negative)	0.066 (–)	$<2.5 \times 10^2$	160	<10	10
G.M.H. (F/29)	HD2 IIAa (EBV-negative)	0.086 (–)	$<2.5 \times 10^2$	40	<10	20
B.C. (F/34)	HD2 IIIAa (EBV-negative)	0.022 (–)	$<2.5 \times 10^2$	40	<10	80
K.Z. (F/28)	HD2 IVBb (EBV-negative)	0.268 (–)	$<2.5 \times 10^2$	320	<10	20
O.T. (M/33)	HD2 IIAa (EBV-negative)	0.178 (–)	$<2.5 \times 10^2$	80	<10	5
D.Y. (F/41)	HD2 IIBb (EBV-negative)	0.229 (–)	$<2.5 \times 10^2$	160	40	80
M.S. (M/23)	HD2 IVBb (EBV-negative)	0.000 (–)	$<2.5 \times 10^2$	160	<10	80

[a]HD2: Hodgkin's disease subtype 2 (nodular sclerosis); HD3: Hodgkin's disease subtype 3 (mixed cellularity).

for statistical comparisons of EBV-negative and EBV-positive patients (a probability of <0.05 was taken as statistically significant). PCR products were submitted to electrophoresis on agar gels and transferred to a nylon membrane to check the specificity of the amplimers by Southern blotting [Laroche et al., 1995]. The specimens were blinded as to serological, pathological, and PCR results as well as to patient information. To minimize experimental variations, the samples were amplified, electrophoresed, and hybridized in a single experiment. For each PCR experiment, a test was performed to detect in the ethidium bromide gel the putative coexistence of a β globin product signaling the release of cellular DNA in the serum sample.

Virus Isolation

Lymphocytes isolated from peripheral blood with Ficoll-Hypaque were cultured until spontaneous outgrowth in complete culture medium supplemented with 10% fetal bovine serum containing 1 μg of cyclosporine A per 3×10^6 cells.

RESULTS

Twelve out of the 30 Hodgkin's disease patients investigated here showed the presence of Reed-Sternberg cells positive for EBER oligoprobes and anti-LMP1 antibodies. None of these patients contained tumor cells positive for EBV replicative gene products as assessed by in situ hybridization using BHLF1/Not probes (0/12) and anti-ZEBRA antibodies (0/4). A single case contained one BHLF1-positive cell that corresponded to a small nonneoplastic lymphocyte.

Anti-ZEBRA IgMs were never detected in this survey. Anti-ZEBRA IgGs were detected in 7/12 EBV-positive cases and only in 4/18 EBV-negative cases (Table I). High antibody titers were observed in five cases: two patients (C.L. and B.M.) with EBV-positive mixed celllularity type and three others (A.R., C.F., and M.J.) with EBV-negative nodular sclerosis (Fig. 1, Table I). It is noteworthy that the highest titers of anti-ZEBRA IgGs were associated with high viral loads (up to 10^7 EBV genome equivalents/ml of serum) and concerned patients with EBV-positive Hodgkin's disease mixed cellularity type. One patient who had the highest anti-ZEBRA antibody titer (with a high viral load of 1.7×10^5 genome equivalents/ml) suffered also from chronic lymphocytic leukemia. In 13/18 EBV-negative cases (11 with undetectable anti-ZEBRA antibodies), the EBV load was $<2.5 \times 10^2$ genome equivalents/ml), whereas 2 patients had a moderate viral load

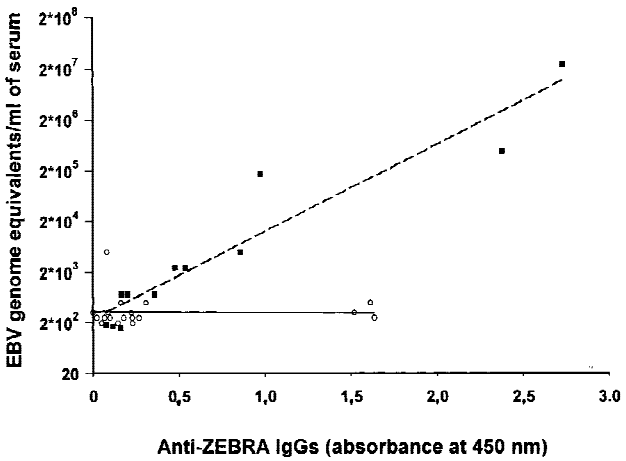

Fig. 1. Correlation between the presence of viral DNA and of anti-ZEBRA IgGs in serum from tumor-bearing patients. Open circle: patients with EBV-negative tumors; filled square: patients with EBV-positive tumors.

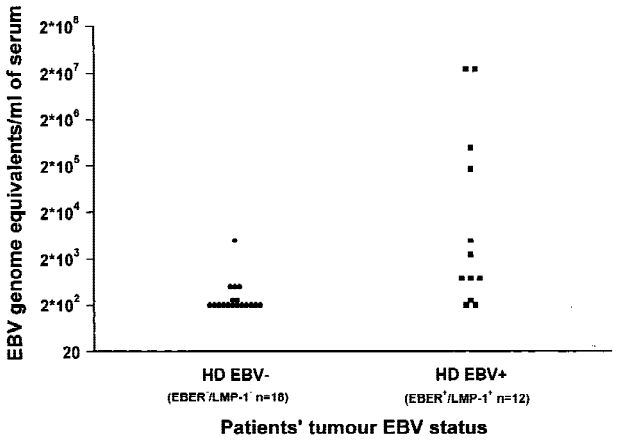

Fig. 2. EBV load from patients with Hodgkin's disease according to the tumor EBV status.

(2.5×10^2/ml and 2.5×10^3/ml) associated with detectable anti-ZEBRA antibodies. There was a significant association between the EBV load in serum and the presence of EBV products (EBER and LMP-1) in tumor cells ($P < 0.04$) as illustrated by Figures 1 and 2.

Overall, there was a positive correlation between the occurrence of viremia and increased IgG anti-ZEBRA titers and/or standard serological profiles of EBV reactivation ($P < 0.05$) regardless of the EBV status of the tumor.

Furthermore, the highest viral loads gave rise to lymphocyte immortalization in vitro as illustrated in Figure 3. PCR products were strongly detected, after gel electrophoresis and Southern transfer, in samples both containing the highest viral loads and giving rise to positive cultures.

DISCUSSION

One of the first studies on the relationship between EBV and Hodgkin's disease reported that an increased risk of Hodgkin's disease was associated with elevated titers of anti–early antigens IgGs, suggesting that the disease could be preceded by a state of EBV reactivation and that deregulation of the EBV-host balance had preceded the outgrowth of a malignant clone [Mueller et al., 1989]. Other data support the implication of lytic cycle activation (through ZEBRA protein) as a source of infection of bystanders B-cells, thereby inducing the outgrowth of lymphoblastoid populations [Rochford and Mosier, 1995].

The results of the present study, describing high anti-ZEBRA antibody levels and the detection of EBV DNA in serum, are in line with the former report. They suggest that in patients with overt Hodgkin's disease, the EBV reactivation state persists after the onset of the disease. These findings are comparable to those observed during infectious mononucleosis where cell-free EBV DNA in serum samples has also been detected and correlated with a serological profile of EBV reactivation [Gan et al., 1994; Laroche et al., 1995; Yamamoto et al., 1995]. The high viral loads measured in patients with EBV-positive Reed-Sternberg cells might be explained by the release of viral genomes from tumor cells through a mechanism of apoptosis. However, these tumor cells did not show any positivity for late antigens. Release of EBV from B-cells in vivo as well as in vitro after cell lysis during clot formation cannot be excluded completely. But among the three patients with EBV-positive Hodgkin's disease subtype 3 and harboring the highest viral loads, two did not exhibit any β globin signal on electrophoresis gels. The latter phenomenon is especially clear for patients with disease at stage IVB, who exhibited the highest viral loads (up to 10^7 genome equivalents/ml). These results are in accord with other reports describing the presence of cell-free EBV DNA in serum and plasma during infectious mononucleosis [Gan et al., 1994; Yamamoto et al., 1995].

In patients with disseminated disease, the high level of EBV DNA could be explained either by the great tumor mass-releasing viral components (through apoptosis), or by an increased immune deficiency allowing the activation of EBV replication in circulating B-cells. This observation on the link between the EBV load and the tumor mass may be reinforced by the frequent association with high anti-ZEBRA titers. A recent work reported that anti-ZEBRA antibodies were detected in 39% and 50% of patients with non-Hodgkin's lymphomas and Hodgkin's disease, respectively. The prevalence of anti-ZEBRA antibodies was marginally superior in patients with increased serum LDH, which is accepted as an indirect measure of the tumor burden [Blay et al., 1996]. As previously reported, EBV replication occurs rarely in EBV-positive RS cells [Brousset et al., 1993a]; in the present study, only one case contained a single nonneoplastic small lymphocyte positive for BHLF1/Not probe. Thus, high anti-ZEBRA titers (and in some cases anti-EA and anti-VCA detected by standard serological methods) observed in our pa-

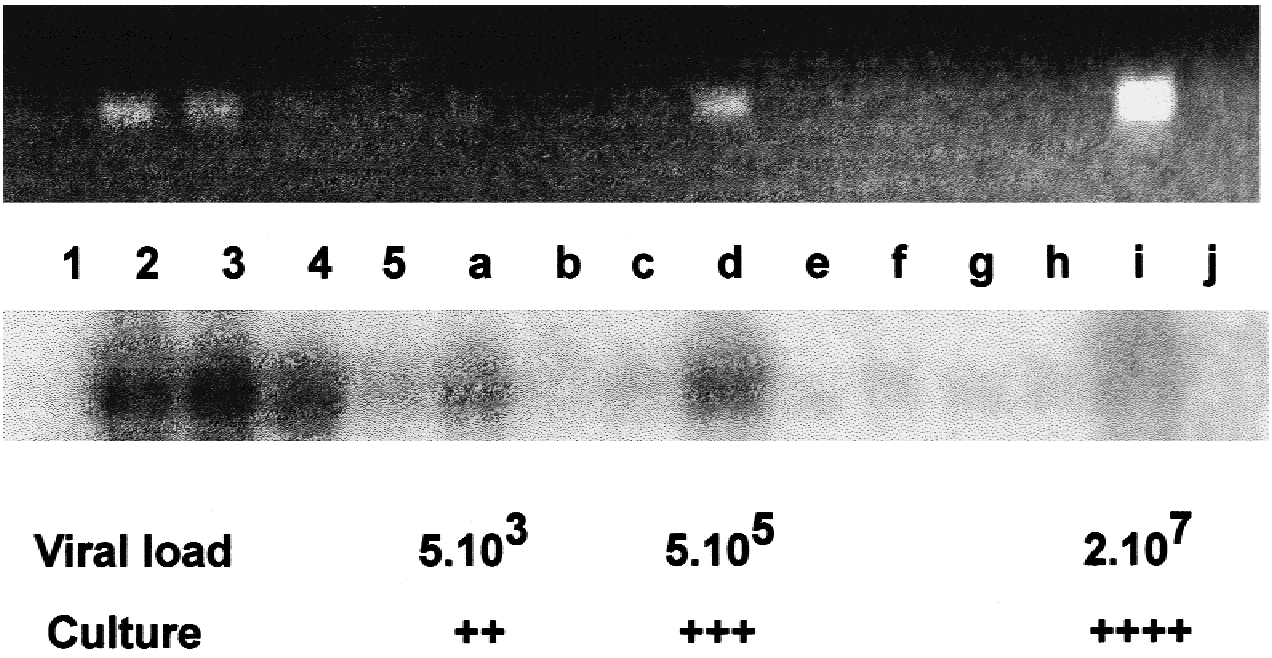

Fig. 3. Correlation between the results of lymphocyte cultures and PCR experiments. Lanes 1–5: samples containing 0, 20,000, 2,000, 200, and 20 viral copies, respectively. Lanes a–j: patient samples. Viral loads were calculated after PCR ELISA as described [Niveleau et al., 1994]. Lanes b, c, e, f, g ,h, and j correspond to patients for which cell culture was negative.

tients cannot be satisfactorily explained by activation of EBV replication in tumor cells.

In addition, comparable titers were observed in Hodgkin's diseases with EBV-negative Reed-Sternberg cells (Table I). Therefore, it appears that EBV replication and viremia detected in Hodgkin's disease patients must originate in other sites than tumor cells. These suspected sites should be the places where high levels of replication occur. Although the precise lineage of the malignant cells in Hodgkin's disease remains undefined, it is possible that increases in ZEBRA levels during de novo infection in nonmalignant cells may cause a sustained binding of ZEBRA to cellular AP-1 sites and inappropriate activation of some cellular genes [Kelleher et al., 1996] leading to malignant transformation. The reservoir of EBV-positive B-lymphocytes might play a role explaining this phenomenon [Niedobitek and Young, 1994]. The immunodeficiency state reported in this lymphoproliferative disorder was not well characterized [Masucci et al., 1984; Gruus et al., 1997].

However, activation of EBV replication in nonneoplastic cells likely occurs as a consequence of the impaired immunity of these patients. Such a hypothesis is reinforced by the presence of high levels of anti-ZEBRA antibodies together with the high EBV DNA loads in the serum obtained from the patient who had also a chronic lymphocytic leukemia, a disease known to be associated with important deregulation of humoral and cellular immunity. Although Hodgkin's disease patients frequently suffer from an impaired immune system, they are often able to mount high anti-ZEBRA antibody levels, as clearly shown here and in another study [Blay et al., 1996], as well as high anti–LMP-1 titers [Chen et al., 1992], both indicating an intact humoral response against these antigens. Concerning impaired immunity in Hodgkin's disease patients, it was also shown that natural inhibitors of T-cell activation did exist in patients with Hodgkin's disease [Roux et al., 1991] and that in EBV-associated Hodgkin's disease cases, tumor-specific factors may elicit a localized suppression of EBV-specific cellular immunity and thus contribute to the pathogenesis of EBV-positive Hodgkin's disease [Frisan et al., 1995]. Recently, Herbst et al. [1996] showed an association between EBV infection and IL-10 expression in H-RS cells, suggesting a potential mechanism to explain this phenomenon. It is possible that, in a context of global impaired immunity, activation of EBV replication in nonneoplastic cells occurs, leading to the expression of ZEBRA and to a rise of anti-ZEBRA antibodies, which are an indirect marker of EBV replication activation.

Still, several questions can be raised about the interaction between ZEBRA and the immune system. First, ZEBRA is an immunogenic protein, of which several B- and T-epitopes have been characterized [Bogedain et al., 1995; Cheng et al., 1995]. Second, this immunity can be deleterious in some clinical situations: a strong correlation exists between ZEBRA and IL-10 expression (human viral IL-10) in nonimmunocompromised individuals with non-Hodgkin's lymphoma [Voorzanger et al., 1994].

Regarding EBV latency profile, Hodgkin's disease and nasopharyngeal carcinoma are comparable and display the *Bam*H1 F promoter-driven latency II form

of EBV infection [Ambinder et al., 1996]. In addition, it has been reported that 87% of nasopharyngeal carcinoma-bearing patients exhibited significant anti-ZEBRA titers, contrasting with the scarcity of tumor cells showing a replicative gene expression [Martel-Renoir et al., 1995]. However, the absence of detectable viremia in nasopharyngeal carcinoma patients [Gan et al., 1994] suggests that, in contrast with Hodgkin's disease, the EBV replicative cycle is mainly incomplete, even in the reservoir of nonneoplastic infected cells.

In conclusion, it was demonstrated that anti-ZEBRA antibodies can be detected in most patients with EBV-associated Hodgkin's disease as well as in a limited number of individuals with EBV-negative Hodgkin's disease and that there is a positive correlation with viremia. These facts may shed new lights on both the pathogenesis of Hodgkin's disease and the management of the disease. Anti-ZEBRA antibodies are an indirect marker of activation of EBV replication, indicating that BZLF-1 gene product is synthesized in infected cells in such patients and that it may accompany the establishment of the transformed phenotype. Furthermore, high levels of anti-ZEBRA antibodies can be used as biological markers in the follow-up of nasopharyngeal carcinoma [Yip et al., 1994] or NHL [Blay et al., 1996] patients. EBV is present in the tumor cell population of up to 50% Hodgkin's disease cases in Western countries [Herbst, 1996], therefore it can be reasonably assumed that an increase of developing Hodgkin's disease can follow infectious mononucleosis as shown by seroepidemiological investigations. Also, anti-ZEBRA titers, together with the quantitation of EBV DNA in serum, can be useful for monitoring Hodgkin's disease patients.

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